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Characterization of the Calmodulin-binding and Catalytic Domains in Skeletal Muscle Myosin Light Chain Kinase*

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Limited proteolysis has been utilized to study the structural organization of rabbit skeletal muscle myosin light chain kinase. The enzyme (M_r ~ 89,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) consists of an amino-terminal, protease-susceptible region of unidentified function and a carboxyl-terminal, protease-resistant region of M_r ~ 40,000 containing the catalytic and calmodulin-binding domains. Partial digestion with trypsin produced an intermediate 56,000-dalton fragment and a stable 38,000-dalton fragment, both of which were catalytically active and calmodulin-dependent. Chymotryptic digestion yielded three catalytically active fragments of about 37,000, 36,000, and 35,000 daltons. The M_r = 37,000 fragment was calmodulin-dependent with an apparent affinity equivalent to that of the native enzyme (~1 nM). The 36,000-dalton fragment was also calmodulin-dependent but had a ~200-fold lower apparent affinity. The M_r = 35,000 fragment was calmodulin-independent. These three chymotryptic fragments, had identical amino termini. Nineteen residues were missing from the carboxyl terminus of the calmodulin-independent chymotryptic fragment whereas only 8 or 9 carboxyl-terminal residues were missing from the calmodulin-dependent tryptic fragments. These results suggest that the 11-residue sequence (IAVSAANRFKK) in the carboxyl-terminal region of myosin light chain kinase contributes directly to the binding of calmodulin. This conclusion is in accord with data (Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., and Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 3187–3191) that the carboxyl-terminal, 27-residue CNBr peptide of the native enzyme shows Ca^{2+}-dependent, high affinity binding to calmodulin and that similar calmodulin-binding activity, although detectable in unfractionated CNBr digests of calmodulin-dependent enzyme forms, is much reduced in a CNBr digest of the calmodulin-independent, M_r = 35,000 chymotryptic fragment.

Myosin light chain kinase phosphorylates, in a Ca^{2+}- and calmodulin-dependent manner, one of the light chain subunits of myosin (light chain 2: M_r = 18,500 in skeletal muscle). This phosphorylation is thought to play an obligatory role in the contractile process in smooth muscle and a modulatory role in contraction in skeletal muscle (for reviews, see Refs. 1 and 2). The enzyme exists in a wide spectrum of tissue- and species-specific isozymic forms (1–4). In skeletal muscle, the enzyme has a molecular weight of between 75,000 and 150,000 depending on the species (3, 4). As purified from rabbit skeletal muscle, it is monomeric, with a molecular weight estimated to be ~80,000–91,000 by SDS-PAGE[1] (5–10). However, since values of 70,000–75,000 daltons have been reported by sedimentation equilibrium measurements (8, 10), the values by SDS-PAGE may be anomalously high.

A number of reports have described proteolytic fragments of skeletal muscle myosin light chain kinase retaining catalytic activity (5, 10, 11, 13). In an early observation, Pires and Perry (5) reported that enzymatically active fragments with molecular weights of 50,000 and 30,000 were generated upon storage of the native enzyme at 2 °C. Tanaka *et al.* (11) reported that trypsin treatment converted the enzyme to a state that no longer required Ca^{2+} and calmodulin for activity, although they did not provide physical characterization of the fragment(s) responsible for this activity. In contrast, Waisman *et al.* (12) failed to observe such an effect in similar studies using trypsin, and Mayr and Heilmeyer (10) used trypsin and an endogenous protease in their kinase preparation to generate calmodulin-regulated fragments of M_r ~ 36,000. Srivastava and Hartshorne (13) reported that limited chymotryptic treatment generated a 65,000-dalton fragment whose activity was independent of Ca^{2+} and calmodulin.

Detailed knowledge of the kinetic aspects of the interaction of calmodulin and skeletal muscle myosin light chain kinase is currently available (7, 8, 14). There is, nevertheless, little structural information concerning this interaction and an incomplete or conflicting picture (see data cited above) of the domain structure of the enzyme as analyzed by limited proteolysis. We therefore undertook an investigation of the nature and relative positioning of its catalytic and calmodulin-binding domains. This report represents one of three articles from our laboratories on this topic. In Blumenthal *et al.* (15) we demonstrated that the carboxyl-terminal, 27-residue cyan-

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[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LBTI, lima bean trypsin inhibitor; HPLC, high performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; CaM, calmodulin; T_a, T_b, tryptic fragments of myosin light chain kinase with molecular weights by SDS-PAGE of about 56,000 and 38,000, respectively; C_a37, C_a36, C_a35: chymotryptic fragments of myosin light chain kinase with molecular weights by SDS-PAGE of about 37,000, 36,000, and 35,000, respectively; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

ogen bromide fragment of myosin light chain kinase has the ability to bind calmodulin in a Ca^{2+}-dependent manner and with high affinity. In Takio *et al.* (16) we present the amino acid sequence of the carboxyl-terminal ~60% of the enzyme, a region containing both the catalytic and calmodulin-binding domains. In this article we describe the generation, purification, and enzymatic characterization of a series of catalytically active proteolytic fragments derived from the carboxyl-terminal part of the enzyme. Four of the fragments were found to be calmodulin-regulated whereas one (the smallest chymotryptic fragment) was unresponsive to calmodulin. Amino- and carboxyl-terminal sequence analysis on this latter fragment and on calmodulin-dependent fragments suggested that the carboxyl-terminal 20 residues contain determinants critical for calmodulin-interaction. Thus, the data presented in these three reports support a model in which the carboxyl-terminal half of myosin light chain kinase contains both the catalytic and calmodulin-binding domains with the latter occupying the ~30 amino acid residues at the extreme carboxyl terminus. Taken together, these data provide the first details of the primary structure of a calmodulin-binding domain in a calmodulin-regulated enzyme and may, therefore, aid in the elucidation of the structures of calmodulin-binding sites in other calmodulin-regulated enzymes.

EXPERIMENTAL PROCEDURES

Materials

α-Chymotrypsin, TPCK-treated trypsin, and lima bean trypsin inhibitor (LBTI) were obtained from Worthington. *Staphylococcus aureus* (V_8) protease, bovine serum albumin, and human IgG were from Miles Laboratories. Leupeptin was purchased from Peninsula Laboratories. Phenylmethylsulfonyl fluoride and pancreatic trypsin inhibitor were obtained from Sigma. [γ-^{32}P]ATP was supplied by New England Nuclear. All other chemicals used were reagent grade or better. Myosin light chain kinase was prepared from rabbit skeletal muscle by a large-scale method described in detail elsewhere (16). Mixed myosin light chains from rabbit skeletal muscle were prepared by a published method (7). The procedure for removing endogenous calmodulin from the light chains was as described (9) with minor modifications. Bovine brain or erythrocyte calmodulin was purified by DEAE- and fluphenazine-Sepharose essentially as described by Kakiuchi *et al.* (17). Calmodulin affinity columns were prepared using either CNBr-activated Sepharose 4B (Pharmacia) or Affi-Gel 15 (Bio-Rad) according to manufacturers' instructions with minor modifications.

Myosin Light Chain Kinase Assay

Myosin light chain kinase was assayed basically as described (9). Prior to assay, enzyme dilutions were made in a buffer containing 10 mM MOPS, pH 7, 1 mg/ml bovine serum albumin, 1 mM EDTA, 0.1 M NaCl, and 0.5–1.0 mM DTT (buffer A). Assays were performed either with mixed myosin light chains or with calmodulin-free mixed myosin light chains for the determination of activation constants. The concentration of the phosphorylatable light chain in these assays (~50 μM) was determined by maximal ^{32}P incorporation. Assays performed in the presence of Ca^{2+} and calmodulin contained saturating concentrations of both (0.4 mM Ca^{2+} in excess of chelator and ≧1 μM calmodulin). Assays performed in the absence of Ca^{2+} and calmodulin contained no added calmodulin and EGTA ≧ 2 mM in excess of Ca^{2+}.

Preparation of Calmodulin-dependent Tryptic Fragments (T_β, T_α/T_β)

T_β—myosin light chain kinase (3.6 mg) in 60 mM MOPS, pH 7.2, 2 mM EGTA, 1 mM DTT, 0.2 M NaCl was incubated at 22 °C with 3.1 μg of TPCK-treated trypsin in a final volume of 2.64 ml. At various times, separate sets of aliquots were removed for SDS-PAGE and myosin light chain kinase activity determinations. LBTI was added to each aliquot to terminate proteolysis (LBTI/trypsin: 34/1, w/w). The aliquots for activity measurements were also diluted 10-fold in buffer A. All aliquots were frozen on dry ice immediately after additions and then stored at −70 °C prior to analysis. The control reaction was performed and processed identically but without protease. After 20 min, an aliquot (3.36 mg of myosin light chain kinase) was removed and LBTI added to terminate proteolysis (LBTI/trypsin: 34/1, w/w). The mixture was then applied to a Sephacryl S-200 (Pharmacia) column (1 × 115 cm) equilibrated in 10 mM MOPS, pH 7, 0.5 mM EDTA, 2.5 mM DTT, and 0.1 M NaCl and chromatographed (4 °C) at a flow rate of 5.8 ml/h. From the 1.4-ml column fractions, separate aliquots were removed for SDS-PAGE and myosin light chain kinase activity measurements after dilution in buffer A. T_β is defined as the pool of S-200 fractions 41–46.

T_α/T_α—A mixture of tryptic fragments (α/β: 1.8/1.0 by sequence analysis) was prepared by treating 3 mg of myosin light chain kinase in 5 mM MOPS, pH 6.5, 5 mM DTT, and 0.1 M NaCl with TPCK-treated trypsin (3 μg) at 22 °C for 30 min in a final volume of 4.29 ml. The digestion was terminated by a 70-fold weight excess of LBTI or in the case of utilization for sequence analysis by the addition of trichloroacetic acid to a final concentration of 10% (w/v).

Preparation of Calmodulin-dependent Chymotryptic Fragments ($C_\beta 37$, $C_\beta 36$)

Myosin light chain kinase (1.94 mg) in 5 mM MOPS, pH 7, 0.5 mM EDTA, 1 mM DTT, and 0.3 M NaCl was incubated at 22 °C with 8.4 μg of chymotrypsin in a final volume of 4.28 ml. At various times aliquots were removed, added to phenylmethylsulfonyl fluoride (0.5 mM, final concentration) to stop proteolysis, frozen on dry ice, and stored at −70 °C for subsequent analysis by myosin light chain kinase assay and SDS-PAGE. The control reaction was performed and processed identically but without addition of protease. After 10 min, to an aliquot containing 1.87 mg of myosin light chain kinase, phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM. The mixture was then applied to a Sephacryl S-200 gel filtration column (1.6 × 92 cm) equilibrated in 5 mM MOPS, pH 7, 0.5 mM EDTA, 2 mM DTT, and 0.1 M NaCl and chromatographed (4 °C) at a flow rate of 14 ml/h. From the 1.3-ml column fractions, aliquots were removed for SDS-PAGE and myosin light chain kinase assay. Fractions 76–83 were pooled (10.4 ml). After removal of an 0.7-ml aliquot, the remainder was adjusted with $CaCl_2$ to a final concentration of 1 mM and applied to a calmodulin-Sepharose column (4 °C; 0.7 × 8.5 cm; coupling density: ~0.25 mg of calmodulin/ml of resin) equilibrated in 10 mM MOPS, pH 7, 100 μM $CaCl_2$, 1 mM DTT, and 0.1 M NaCl. The column was then washed with an additional 2.1 ml of this buffer and the flow-through collected in three fractions. Flow-through fractions 1 and 2 were combined and are termed $C_\beta 36$. The third flow-through fraction contained a minimal amount of protein and was discarded. Elution was with the equilibration buffer containing 1 mM EDTA. Those fractions in the eluate containing protein were pooled and designated $C_\beta 37$.

Preparation of the Calmodulin-independent Fragment ($C_\beta 35$)

Myosin light chain kinase (8.63 mg) in 90 mM MOPS, pH 7.2, 0.5 mM EDTA, 1 mM DTT, 0.1 M NaCl was incubated at 22 °C with 43 μg of chymotrypsin in a final volume of 2.09 ml. At various times, separate sets of aliquots were removed for SDS-PAGE and myosin light chain kinase activity determinations. LBTI was added to each aliquot to terminate proteolysis (LBTI/chymotrypsin: 34/1, w/w). To the aliquots for activity measurements, 0.9 mg/ml bovine serum albumin was also added. All aliquots were frozen on dry ice immediately after additions and then stored at −70 °C prior to analysis. The control reaction was performed and processed identically but without protease. After 20 min, an aliquot containing 6.86 mg of myosin light chain kinase was removed, and chymotrypsin inactivated with a 20-fold weight excess of LBTI. The mixture was then subjected to HPLC gel filtration (22 °C) at a flow rate of 2.5 ml/min on a Bio-Sil TSK-250 preparative column (G3000SW; 21.5 × 600 mm), equilibrated in 10 mM MOPS, pH 6.8, 0.5 mM EDTA, 1 mM DTT, and 0.1 M NaCl. Fractions containing activity were pooled and adjusted with $CaCl_2$ to a final concentration of 1 mM. The pool was applied to a column (4 °C; 0.7 × 4.2 cm) of calmodulin immobilized on Affi-Gel-15 (coupling density: ~1 mg of calmodulin/ml resin) equilibrated in 10 mM MOPS, pH 7, 100 μM $CaCl_2$, 1 mM DTT, and 0.1 M NaCl. The column was then washed with an additional 2.5-column volumes of the equilibration buffer and elution was with this buffer containing 2 mM EGTA substituted for $CaCl_2$. Protein containing flow-through fractions (2–10) were pooled and designated $C_\beta 35$.

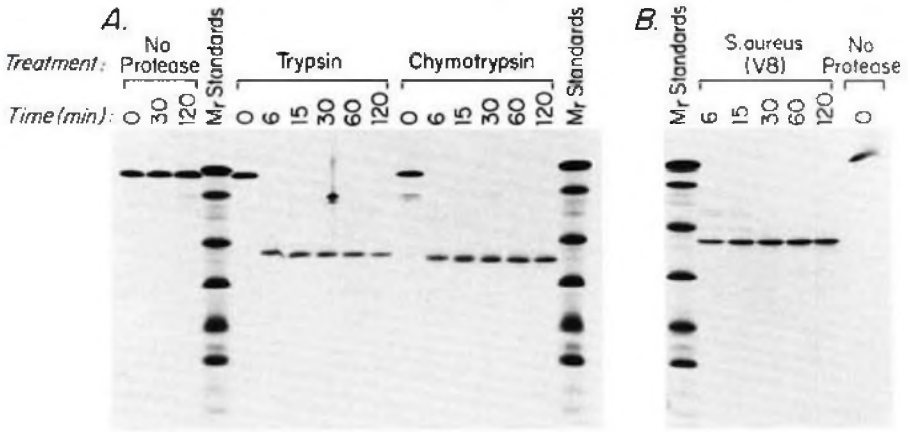

FIG. 1. **Limited digestion of myosin light chain kinase with trypsin, chymotrypsin, or *S. aureus* (V_8) protease.** Myosin light chain kinase (96 μg/150-μl reaction) was incubated on ice in separate reactions with trypsin (2.0 μg), chymotrypsin (2.3 μg), or *S. aureus* (V_8) protease (5.8 μg). The reactions also contained 0.08 M Tris, pH 7.5, 0.01 M MOPS, pH 7, 0.08 M NaCl, 0.8 mM DTT, 0.8 mM EDTA, 8% (v/v) glycerol. At the indicated times, aliquots (4.4 μg from the trypsin and chymotrypsin incubations, and 5.5 μg from the *S. aureus* (V_8) protease incubation) were added to protease inhibitor mixtures containing (in final concentrations): pancreatic trypsin inhibitor (15 μg/ml), leupeptin (0.11 mM) phenylmethylsulfonyl fluoride (2.8%, w/v), and ethanol (2.6%, v/v). Samples were then prepared for and subjected to SDS-PAGE as described under "Experimental Procedures." *A* and *B* represent separate gels. For the zero time points trypsin, chymotrypsin, or H_2O (no protease condition) were preincubated with the protease inhibitor mixture before addition to the kinase.

TABLE I

Preparation of Ca^{2+}-calmodulin-dependent enzyme fragments (T_s, C_s37, C_s36)

Step	Protein	Total activity	Specific activity	Calmodulin dependence[a]
	mg	*μmol/min*	*μmol/min/mg*	*%*
A. Tryptic fragment				
Intact myosin light chain kinase	3.30	27.2	8.24	99.5
S-200 pool (Fractions 41–46:T_s)	0.857	6.85	7.99	99.6
B. Chymotryptic fragments				
Intact myosin light chain kinase	1.64	12.24	7.47	99.6
S-200 Pool	0.476	4.84	10.20	98.5
Calmodulin affinity column flow through (C_s36)	0.332	3.09	9.31	100.0
Calmodulin affinity column chelator eluate (C_s37)	0.029	0.332	11.45	98.4

[a] Calmodulin dependence (%) is defined as:

$$\left(1 - \frac{\text{Activity in the absence of calmodulin and presence of EGTA}}{\text{Activity in the presence of saturating concentrations of } Ca^{2+} \text{ and calmodulin}}\right) \times 100.$$

Selective Cleavage and Sequence Analysis

Intact myosin light chain kinase and fragments of limited proteolysis were reduced, and carboxymethylated as described by Takio *et al.* (18), then acidified and separated from the reagents and contaminating smaller fragments on TSK gel filtration columns as described (see Fig. 11, legend). Pooled fractions were desalted on a Sephadex G-25 column in 9% (v/v) formic acid and lyophilized. Methionyl bonds were cleaved with 1% (w/v) CNBr in 70% (v/v) formic acid (~10 mg/ml protein) for 6 h at room temperature. Primary separation of CNBr fragments was achieved by TSK gel filtration as described in the legend to Fig. 11. Amino acid analysis was performed with a Dionex D500 Amino Acid Analyzer or a Waters Pico-Tag system (19, 20). Automated Edman degradations were carried out on a gas-phase protein sequencer (model 470A, Applied Biosystems) with a program adapted from Hunkapiller *et al.* (21). Phenylthiohydantoins were identified in a semiquantitative manner by two complementary HPLC systems (22, 23).

Gel Electrophoresis

Samples were prepared by heating at >90 °C for 3–5 min in 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, and 8% (v/v) glycerol. SDS-PAGE was performed as described (24) using either 20% acrylamide, 0.08% bisacrylamide or 15% acrylamide, 0.15% bisacrylamide in the running gels. The following M_r standards (Bio-Rad) were used: phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (44,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Other Methods

Protein concentrations were determined as described by Bradford (25), using bovine serum albumin as standard, or by the method of Lowry *et al.* (26), with some of the modifications suggested by Peterson (27), using human IgG as standard. Calmodulin concentrations were measured by UV absorbance using $E^{1\%}_{280} = 2.0$ (28).

RESULTS

Isolation and Characterization of Enzymatically Active Fragments of Myosin Light Chain Kinase Generated by Limited Proteolysis—Initial experiments showed that when purified rabbit skeletal muscle myosin light chain kinase was subjected to limited proteolysis the fragmentation pattern obtained was largely independent of the type of protease used (Fig. 1, *A* and *B*). *S. aureus* (V_8) protease, trypsin, or chymotrypsin, all produced a similar pattern as analyzed by SDS-PAGE. The enzyme (M_r = 89,000) was rapidly degraded to an M_r = 35,000–40,000 fragment which was largely resistant to further proteolysis suggesting a compact structure. Amino-terminal se-

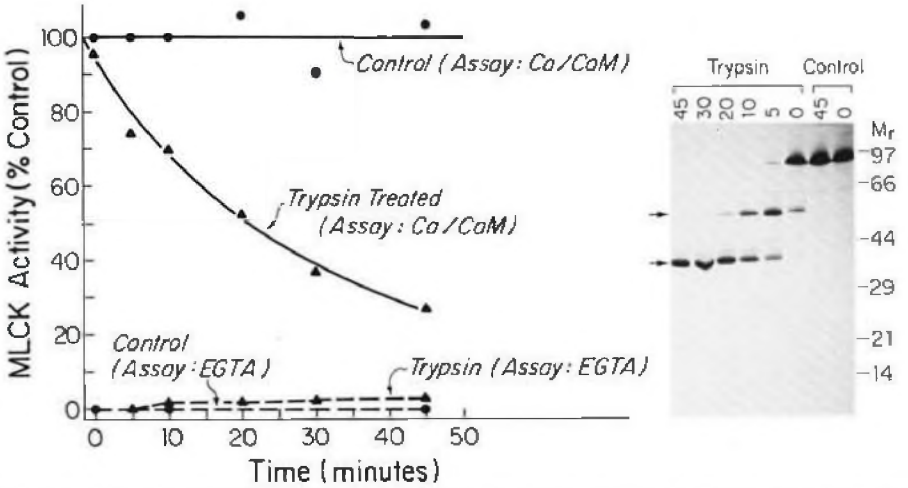

FIG. 2. **Limited tryptic digestion of myosin light chain kinase (MLCK).** Myosin light chain kinase was subjected to limited digestion with TPCK-treated trypsin as described under "Experimental Procedures" (T_β preparation). *Left panel*, kinase assay of selected time points from the following reactions: trypsin treated, assayed with Ca^{2+}, calmodulin (▲——▲); no protease, assayed with Ca^{2+}, calmodulin (●——●); trypsin treated, assayed without Ca^{2+}, calmodulin (EGTA included) (▲- - -▲); no protease, assayed without Ca^{2+}, calmodulin (EGTA included) (●- - -●). *Right panel*, SDS-PAGE of selected time points. Times are shown above the gel in minutes. Zero time points in this case represent ~20 s of incubation with, or without (control), trypsin. Each lane contained 8.4 μg. Molecular mass values are in kilodaltons. *Arrows* indicate positions of T_α and T_β.

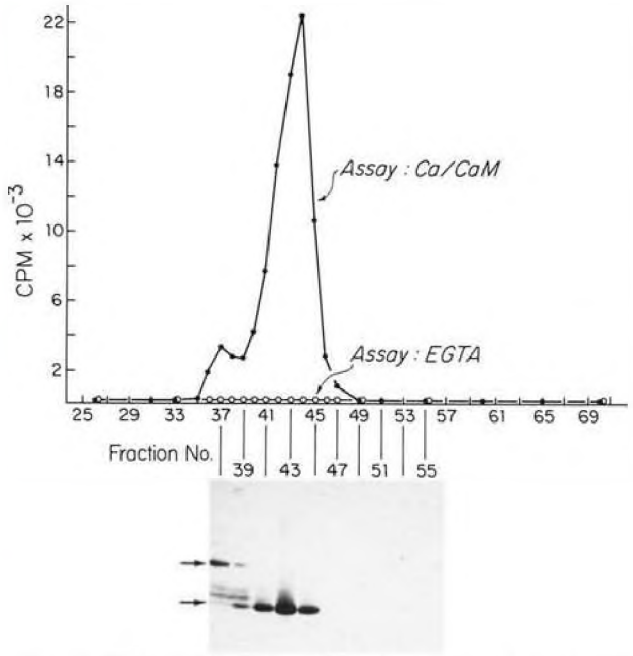

FIG. 3. **Sephacryl S-200 gel filtration chromatography of a limited tryptic digest of myosin light chain kinase.** After 20 min of limited tryptic digestion (Fig. 2), T_α and T_β were separated by Sephacryl S-200 gel filtration chromatography as described under "Experimental Procedures" (T_β preparation). Myosin light chain kinase activity (shown in the *upper panel*) was determined in the presence (●) or absence (○) of Ca^{2+} and calmodulin (EGTA included) as described under "Experimental Procedures." SDS-PAGE of 60-μl aliquots of fractions 37–45 (alternate fractions) are shown in the *lower panel*. Fractions 29–35 and 47–71 were also electrophoresed but showed no protein staining and are therefore not shown. *Arrows* indicate the positions of T_α and T_β.

quence analysis established that these fragments correspond to the carboxyl-terminal ~50–60% of the molecule (16). The lack of obvious stained peptides representing the amino terminal part of the molecule suggested that by contrast, the latter has an extended structure and is therefore quickly digested or very poorly stained by Coomassie Blue.

TABLE II

Activation constants (K_{act}) for calmodulin of isolated forms of skeletal muscle myosin light chain kinase

Enzyme form	K_{act}[a]
	nM
Intact	0.9
Tryptic	
T_α/T_β	11
T_β	28
Chymotryptic	
$C_\beta 37$	0.5
$C_\beta 36$	118
$C_\beta 35$	NA[b]

[a] Defined as the calmodulin concentration required for half-maximal activation.

[b] NA, non-activated; tested at a calmodulin concentration of 5 μM.

Milder treatment with trypsin revealed a fragment of M_r ~ 60,000 as well as the stable M_r ~ 40,000 fragment (Fig. 2). The appearance and disappearance of these fragments with time, as well as their enzymatic and primary structural characteristics (discussed below and in Takio *et al.* (16)), suggested that the M_r ~ 60,000 fragment is further degraded to form the M_r ~ 40,000 fragment. These tryptic fragments are referred to here and in Takio *et al.* (16) as T_α and T_β, respectively. They were previously referred to as T60 and T40, respectively (15). The molecular weights of T_α and T_β are ~50,000 and 41,006 by their amino acid sequences (16) or 55,800 and 38,200 by SDS-PAGE, respectively. Fig. 2 shows that after 10 min of digestion, although there was no intact kinase left, a considerable amount of activity (70%) remained, demonstrating that the fragments were collectively, catalytically active. By 20 min only T_β is evident and is catalytically active. Some activity is lost during the digestion suggesting that the remainder of the molecule may be required for the maintenance of maximal activity. At all time points throughout the digestion the fragments were Ca^{2+}-calmodulin-de-

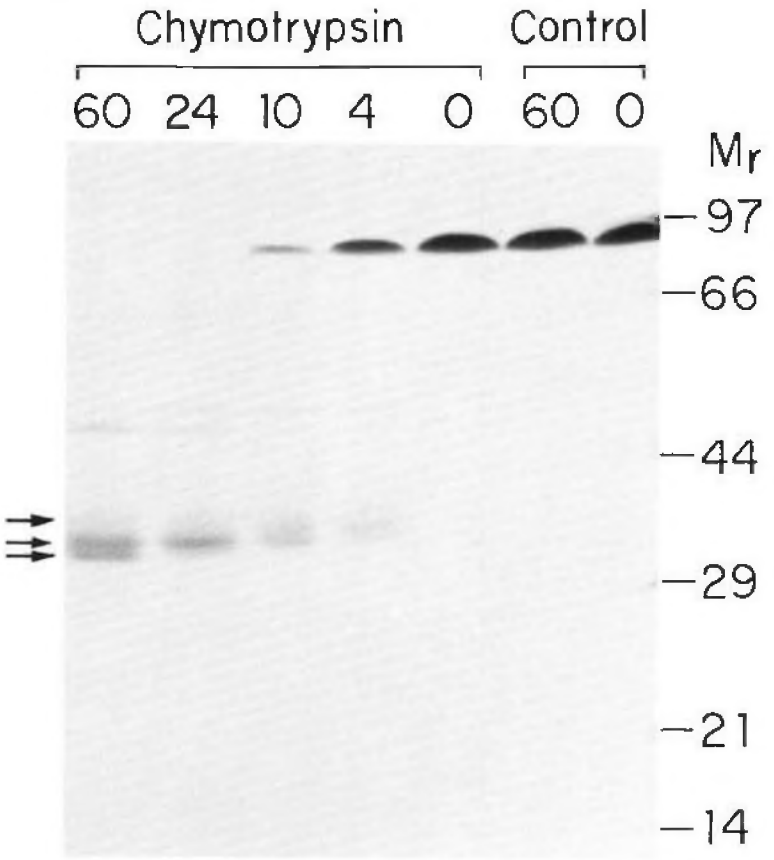

FIG. 4. **SDS-PAGE of a limited chymotryptic digestion of myosin light chain kinase.** Myosin light chain kinase was subjected to limited chymotryptic digestion and aliquots (3.1 μg) taken at selected time points were analyzed by SDS-PAGE ("Experimental Procedures:" $C_\beta 37$, $C_\beta 36$ preparation). The time points selected are shown above the gel in minutes. For the zero time points the order of addition of phenylmethylsulfonyl fluoride and protease (or H_2O for the control reaction) was reversed. Molecular mass values are in kilodaltons. *Arrows* indicate the positions of $C_\beta 37$, $C_\beta 36$, and $C_\beta 35$.

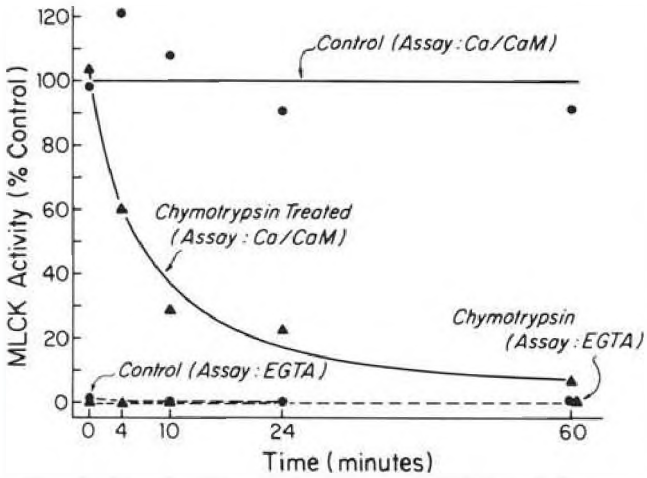

FIG. 5. **Myosin light chain kinase (MLCK) activity as a function of time during limited chymotryptic digestion.** Myosin light chain kinase was subjected to limited chymotryptic digestion as described under "Experimental Procedures" ($C_\beta 37$, $C_\beta 36$ preparation). Myosin light chain kinase activity was determined at selected time points from the following reactions: chymotrypsin treated, assayed with Ca^{2+}, calmodulin (▲——▲); no protease, assayed with Ca^{2+}, calmodulin (●——●); chymotrypsin treated, assayed without Ca^{2+}, calmodulin (EGTA included) (▲ – – – ▲); no protease, assayed without Ca^{2+}, calmodulin (EGTA included) (● – – – ●).

pendent. After 20 min an aliquot of the reaction mixture (after the addition of an excess of protease inhibitor) was subjected to Sephacryl S-200 gel filtration as shown in Fig. 3. Both T_α and T_β demonstrated Ca^{2+}-calmodulin-dependent enzyme activities (see also Takio *et al.* (16)). The pool of fractions 41–46 yielded an electrophoretically homogenous preparation of T_β. Table I(A) summarizes the purification of T_β. On a molar basis, its specific activity (304 μmol/min/μmol) was about 40% that of the intact enzyme (733 μmol/min/μmol). Isolated T_β or an unfractionated mixture of T_α and T_β (see "Experimental Procedures") showed modest increases in their activation constants for calmodulin (Table II).

We also examined in greater detail the cleavage pattern obtained with chymotrypsin. At a lower chymotrypsin/kinase ratio than that used in the experiment shown in Fig. 1*A*, three fragments were obtained having molecular weights by SDS-PAGE of 36,700, 35,500, and 34,600 (Fig. 4). In Takio *et al.* (16), we describe the generation of chymotryptic fragments for sequencing purposes. The chymotryptic fragment there termed C_β appears to be a mixture of the ~37-, 36-, and 35-kilodalton fragments shown here. In this report, therefore, we use the terminology $C_\beta 37$, $C_\beta 36$, and $C_\beta 35$. Fragment $C_\beta 35$, earlier termed C35 (15), has a molecular weight by amino acid sequence of 37,657 (Fig. 12 and Takio *et al.* (16)). The fragmentation pattern with time indicated that $C_\beta 37$ gives rise to $C_\beta 36$ which in turn generates $C_\beta 35$. Fig. 5 shows that these fragments collectively possessed calmodulin-dependent myosin light chain kinase activity although there was a somewhat greater drop in overall activity than in the case of the limited tryptic digestion (Fig. 2). After 10 min of digestion, at which time there were approximately equal amounts of $C_\beta 37$ and $C_\beta 36$ (see Fig. 4), an aliquot of the reaction was removed and proteolysis stopped with an excess of protease inhibitor. The resulting fragments were subjected to Sephacryl S-200 gel filtration as described under "Experimental Procedures." The kinase activity eluted from the column in two peaks. One peak corresponded to undegraded enzyme. The other contained both $C_\beta 37$ and $C_\beta 36$ (not shown). The fractions containing the fragments were pooled and subjected to calmodulin affinity chromatography as described under "Experimental

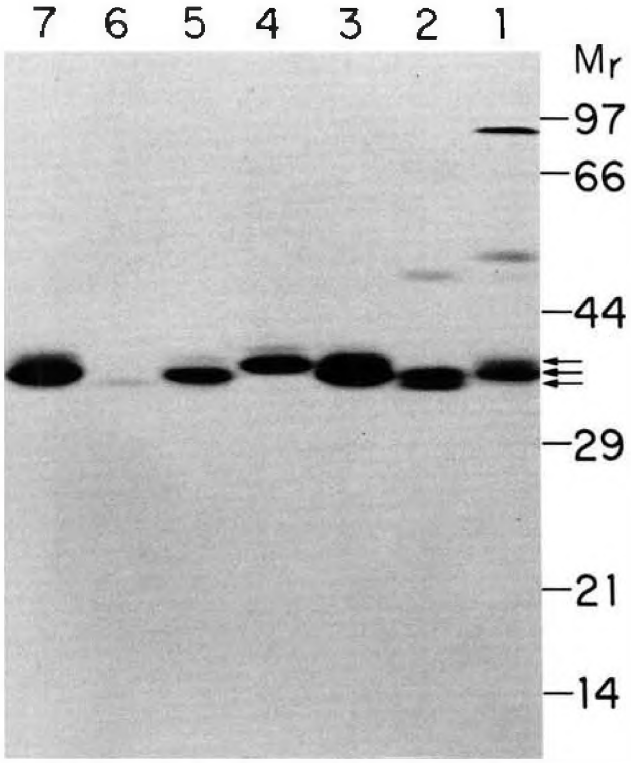

FIG. 6. **SDS-PAGE of purified chymotryptic fragments (C_β37, C_β36) of myosin light chain kinase.** Chymotryptic fragments were generated, purified, and analyzed by SDS-PAGE as described under "Experimental Procedures" (C_β37, C_β36 preparation). *Lane 1*, 10-min time point of original digestion (3.5 μg); *Lane 2*, 60-min time point of original digestion (3.5 μg); *Lane 3*, pool of S-200 fractions 76–83 (3.4 μg); *Lane 4*, EDTA eluate of calmodulin affinity column (1.9 μg); *Lanes 5* and *7*, flow-through fractions 1 and 2 of the calmodulin affinity column (~2.0, ~3.0 μg, respectively); *Lane 6*, calmodulin-independent chymotryptic fragment (C_β35) prepared by a procedure similar to that described under "Experimental Procedures" (C_β35 preparation) (1.1 μg). Molecular mass values are in kilodaltons. *Arrows* indicate the positions of C_β37, C_β36, and C_β35.

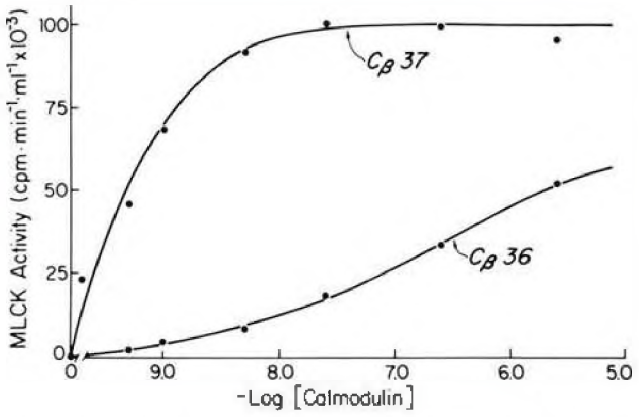

FIG. 7. **Activation of chymotryptic fragments of myosin light chain kinase (MLCK) (C_β37, C_β36) by calmodulin.** Myosin light chain kinase activity was determined as a function of calmodulin concentration for the chymotryptic fragments C_β37 and C_β36, as described under "Experimental Procedures." Calmodulin dilutions were in buffer A.

Procedures." The purified fragments obtained by this procedure were analyzed by SDS-PAGE, the results of which are shown in Fig. 6. Upon application of the mixture of C_β37 and C_β36 obtained by gel filtration (*lane 3*) to the calmodulin-Sepharose affinity column in the presence of Ca^{2+}, C_β36 did not bind (*lanes 5* and *7*) whereas C_β37 did and was specifically eluted with EDTA (*lane 4*). *Lanes 1* and *2* show two time points in the original digestion included to verify the identity of the fragments. As described below, C_β35 could also be isolated in homogenous form. A sample of C_β35 isolated by a similar procedure to that described in detail under "Experimental Procedures" was electrophoresed in *lane 6*. As can be seen, it clearly runs below the bands in *lanes 5* and *7* thereby confirming the latter as C_β36.

Isolated C_β37 and C_β36 (the latter being the pool of fractions shown in *lanes 5* and *7* of Fig. 6) were examined for their abilities to be activated by calmodulin (Fig. 7). Although both fragments had an absolute requirement for calmodulin, C_β36 had a ~200-fold lower apparent affinity for calmodulin than did C_β37. The K_{act} of the latter for calmodulin is equal to that of the native enzyme (Table II). The low apparent affinity of C_β36 for calmodulin and the low coupling density of calmodulin in the batch of affinity resin used probably explains why C_β36 did not bind to the affinity column.

The purification of C_β37 and C_β36 is summarized in Table I(B). As in the case of T_β, the fragments were only moderately reduced in molar specific activity with respect to the intact enzyme and, as was mentioned, were entirely calmodulin-dependent.

Preliminary experiments suggested that the smallest of the three chymotryptic fragments (C_β35) was also enzymatically active but unlike C_β37 and C_β36 was calmodulin-independent (data not shown). In order to generate quantities of C_β35 sufficient for amino acid sequencing and kinetic characterization, the digestion procedure used to generate C_β37 and C_β36 was modified, as described under "Experimental Procedures," by the use of a slightly higher chymotrypsin/kinase ratio and longer time period. Fig. 8 shows the fragmentation and activity patterns during the limited proteolysis. Ca^{2+}-calmodulin-independent activity appeared which roughly coincided with the appearance of C_β35. After 20 min, an aliquot of the reaction was removed, an excess of protease inhibitor added, and the mixture subjected to HPLC gel filtration. Fractions containing appreciable activity were pooled and subjected to calmodulin affinity chromatography. Fig. 9 shows that the protein and virtually all of the activity passed directly through the calmodulin column in the presence of Ca^{2+}. Furthermore, this activity was calmodulin-independent. A very small amount of calmodulin-dependent activity was detected in fraction 15 of the EGTA eluate.

As shown in Fig. 10, the final preparation of C_β35 (consisting of breakthrough fractions from the affinity column) was electrophoretically homogeneous (*lanes 3–5*). *Lanes 1* and *2* are time points in a chymotryptic digestion included to illustrate the relative positions of the three fragments. Although homogenous by SDS-PAGE, some contamination with larger amino-terminal fragments was detected by sequence analysis (discussed below).

The purification of C_β35 is summarized in Table III. Although considerable loss in activity was sustained, the specific activity of the final preparation was still nearly 2 μmol/min/mg. In a replicate generation and purification of C_β35, virtually identical results were obtained (specific activity = 1.75 μmol/min/mg; 0% calmodulin dependence). An unusual aspect of this preparation was that although not entirely Ca^{2+}-calmodulin independent at the point of stopping the digestion, the enzyme was so after the affinity chromatography. A possible explanation for this is presented below (see "Discussion").

Identification of the Termini of the Active Fragments—Since

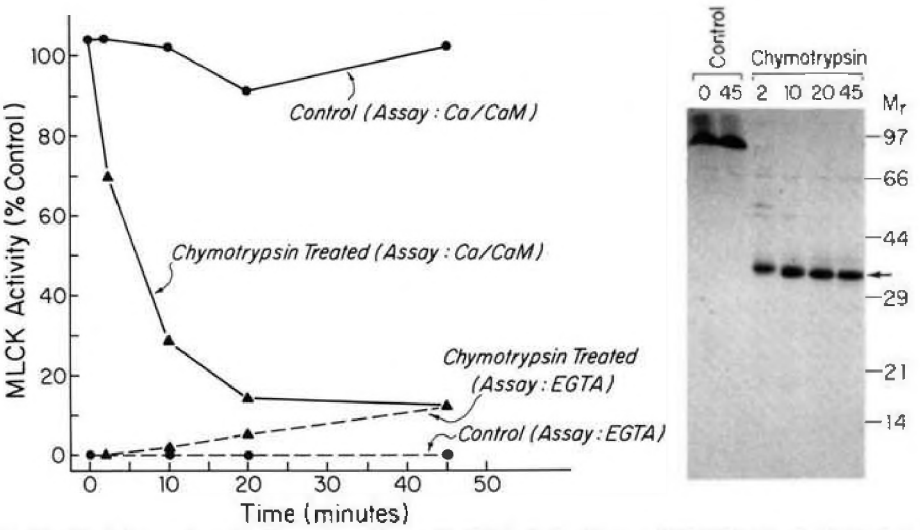

FIG. 8. **Limited chymotryptic digestion of myosin light chain kinase (MLCK).** Myosin light chain kinase was subjected to limited chymotryptic digestion as described under "Experimental Procedures" ($C_\beta 35$ preparation). *Left panel*, kinase assay at selected time points from the following reactions: chymotrypsin-treated, assayed with Ca^{2+}, calmodulin (▲——▲); no protease, assayed with Ca^{2+}, calmodulin (●——●); chymotrypsin-treated, assayed without Ca^{2+}, calmodulin (EGTA included) (▲ - - - ▲); no protease, assayed without Ca^{2+}, calmodulin (EGTA included) (● - - - ●). *Right panel*, SDS-PAGE of selected time points. Times are shown above the gel in minutes. Each lane contained 8.6 μg. Molecular mass values are in kilodaltons. *Arrow* indicates the position of $C_\beta 35$.

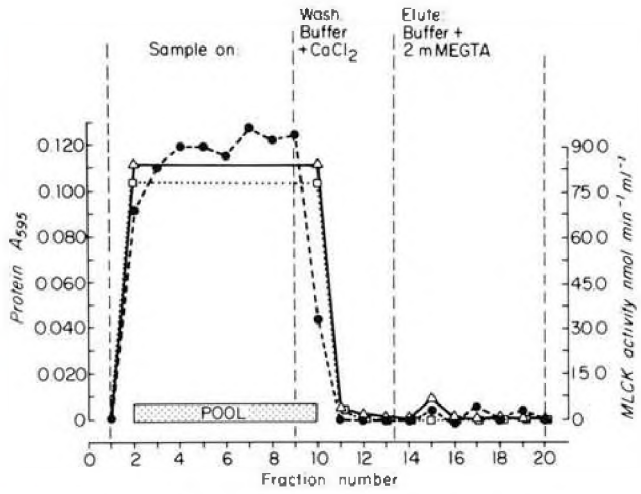

FIG. 9. **Calmodulin affinity chromatography of a chymotryptic fragment ($C_\beta 35$) of myosin light chain kinase (MLCK).** Myosin light chain kinase was subjected to limited chymotryptic digestion and the active fragment purified by sequential HPLC gel filtration and calmodulin affinity chromatography (shown above) as described under "Experimental Procedures" ($C_\beta 35$ preparation). Buffer, 10 mM Mops, pH 7, 1 mM DTT, and 0.1 M NaCl. $CaCl_2$ concentration during sample application and wash was 100 μM. Protein concentrations (● - - - ●) were determined according to the method of Bradford (25). Myosin light chain kinase was assayed as described under "Experimental Procedures," with Ca^{2+}, calmodulin (△——△) or without Ca^{2+}, calmodulin (EGTA included) (□ · · · □).

a very small change (~2200 daltons by SDS-PAGE) in molecular mass converted a Ca^{2+}-calmodulin-dependent enzyme ($C_\beta 37$) to an independent one ($C_\beta 35$) and an even smaller change ($C_\beta 36$ ~ 1200 daltons smaller than $C_\beta 37$) affected the apparent affinity for calmodulin, it was obvious that the amino and/or the carboxyl termini of these fragments was critical for interaction with calmodulin (Table II).

Sequence analysis established the amino termini of the isolated enzyme forms generated by limited proteolysis as: T_α:RGSPA ...; T_β:GIEF ...; $C_\beta 37$:CLPA ...; $C_\beta 36$:CLPA ...; $C_\beta 35$:CLPA.... The fact that the three chymotryptic fragments shared the same amino terminus despite the dramatic differences in the ability to interact with calmodulin (see Table II) is evidence of the importance of the carboxyl-terminal region for this interaction.

Carboxyl-terminal sequence analysis relied on comparisons of fingerprints of the cyanogen bromide fragments from whole protein (Fig. 11, *panel a*) and from the products of limited tryptic digestion (*panel b*) or $C_\beta 35$ (*panel c*). As seen in *panels b* and *c* of Fig. 11, the amino-terminal cyanogen bromide fragment M1 is not present in the digests of T_α/T_β or of $C_\beta 35$. New amino-terminal fragments are denoted M1′ from T_α, M1″ from T_β, and M1^c from $C_\beta 35$. A contaminant in $C_\beta 35$ coeluted with M2. The carboxyl-terminal peptide of the whole protein, M13 in *panel d*, is replaced by M13′ in the digest of T_α/T_β (*panel e*), and by M13^c in the digest of $C_\beta 35$ (*panel h*). In the case of the digest of $C_\beta 35$, a new carboxyl-terminal fragment was suggested by the increase in the relative peak height of M7 (compare *panels a* and *c*), and by the disappearance of M13 peaks upon rechromatography on reversed-phase HPLC (*panel f*). This was confirmed by the characterization of a new peptide M13^c that coeluted with M7 in *panel c* but separated from it in *panel h*. Sequence analysis of the isolated peptides yielded the sequences KRRWKKNFIAVSAANRFKk (M13′) and KRRWKKNF (M13^c), which may be compared with the carboxyl-terminal peptide M13 from the whole protein, KRRWKKNFIAVSAANRFKKISSSGALM (16). Sequence analysis of T_β in the absence of T_α yielded the same carboxyl terminus seen in the mixture (16). Thus, the evidence presented here indicates that the carboxyl terminus of the calmodulin-dependent fragment T_β (or T_α) is only 8 or 9 residues shorter than that of the intact enzyme, whereas the carboxyl terminus of the calmodulin-independent fragment, $C_\beta 35$, is 19 residues shorter than that of the intact enzyme, results consistent with a calmodulin binding domain localized to this region.

Fig. 12 is a schematic representation of myosin light chain kinase showing the sites of proteolytic cleavage and the putative calmodulin-binding domain (M13). Also shown is the

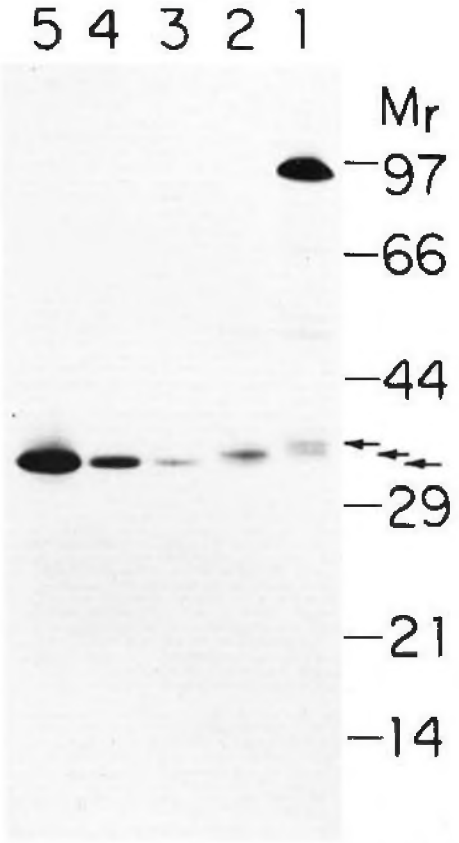

FIG. 10. **SDS-PAGE of a purified chymotryptic fragment (C_β35) of myosin light chain kinase.** C_β35 was generated, purified, and analyzed by SDS-PAGE as described under "Experimental Procedures." *Lane 1*, 4-min time point of chymotryptic digestion shown in Fig. 4 (3.1 μg); *Lane 2*, mixture of 24- and 60-min time points of chymotryptic digestion shown in Fig. 4 (4.0 μg); *Lane 3*, C_β35 (1.1 μg); *Lane 4*, C_β35 (2.2 μg); *Lane 5*, C_β35 (6.2 μg). Molecular mass values are in kilodaltons. *Arrows* indicate positions of C_β37, C_β36, and C_β35.

TABLE III

Preparation of the chymotryptic, Ca^{2+}-calmodulin-independent fragment (C_β35)

Step	Protein	Total activity		Specific activity		Calmodulin dependence[a]
		Ca/CaM	EGTA	Ca/CaM	EGTA	
	mg	*μmol/min*		*μmol/min/mg*		%
Intact myosin light chain kinase	6.58	70.2	0.091	10.7	0.01	99.9
TSK G3000 SW	1.38	3.75	1.97	2.72	1.43	47.5
Calmodulin affinity column flow through	1.47	2.83	2.69	1.93	1.83	4.9

[a] Defined as in Table I.

approximate location of the catalytic domain which can be deduced from the high degree of homology between the sequence of the catalytically active tryptic fragment (T_β) and the catalytic domains of other protein kinases (16).

DISCUSSION

Protein kinases comprise an enzyme family whose members although differing widely in size, specificity, and mode of regulation, appear to have in common at least one important structural feature, namely a marked similarity in their catalytic domains. The catalytic moieties of such distinct kinases as cAMP- and cGMP-dependent protein kinases, γ subunit of phosphorylase kinase, casein kinase II, epidermal growth factor receptor, and $pp60^{src}$ in general consist of either a ~40,000-dalton catalytic subunit or a ~30,000–40,000-dalton catalytic domain residing in the carboxyl-terminal region of the larger molecule (29–37). All of the protein kinase "catalytic units" reported to date exhibit sequence homology (summarized in Takio *et al.* (31)). Skeletal muscle myosin light chain kinase clearly shares these features. Isolated tryptic and chymotryptic fragments of ~30,000–40,000 daltons are enzymatically active (Tables I–III, Ref. 10), the catalytic domain resides in the carboxyl-terminal half of the molecule, and it shows a high degree of homology with the catalytic domains of other protein kinases (16).

Mayr and Heilmeyer (10) have proposed a model for myosin light chain kinase in which the catalytic unit consists of a globular "head" region with a high degree of α-helix, linked to a highly asymmetric, enzymatically inactive, "tail" of low α-helix content. The resistance of the catalytic domain to extensive proteolysis and the rapidity of digestion of the remainder of the molecule (Fig. 1) are consistent with this model. Under certain conditions we have also observed the tail piece and confirm that it is without catalytic activity (data not shown). One possible function for the tail unit would be in aiding the maintenance of a tertiary structure required for optimal enzyme activity. This hypothesis is supported by the partial loss of specific activity accompanying generation of the active fragment (Figs. 2, 5, and 8). This partial loss could not be prevented by the inclusion of MgATP or glycerol in the reaction mixture or by doing the proteolysis on ice rather than at 22 °C. The inclusion of Ca^{2+}/calmodulin during the proteolysis accelerated the loss of activity.[2] Mayr and Heilmeyer (10) report comparable losses in generating their active fragment preparations (specific activities: 13–25% that of intact enzyme) and in addition report the intriguing finding that in at least one case, adding back tail fragment could restore much of the activity lost from the isolated head fragment.

Although the catalytic domains of protein kinases have been studied in detail, there is much less data on the structure of the regulatory sites in these enzymes. For polypeptide-activated protein kinases such as phosphorylase kinase, smooth muscle myosin light chain kinase, epidermal growth factor-, or insulin-receptor kinases, relatively large regions have been identified as regulator binding, although the specific residues involved in this interaction have not been identified (38–42). More is known of the regulatory sites for cyclic nucleotides since both cAMP- and cGMP-dependent protein kinases are homologous with the *Escherichia coli* catabolite gene activator protein that binds cAMP and for which the residues involved in binding have been identified (43, 44). Little is known of the sequence determinants involved in calmodulin binding to enzymes. We present in Takio *et al.* (16), the amino acid sequence of the carboxyl-terminal half of myosin light chain kinase containing both the catalytic and calmodulin-binding domains. The evidence that the latter occupies the ~30 carboxyl-terminal residues is detailed here and in Blumenthal *et al.* (15). This evidence is as follows: 1) the isolated or chemically synthesized carboxyl-terminal, 27-residue peptide M13 binds calmodulin with high affinity as determined by the ability of the peptide to inhibit ($K_i \cong 1$ nM) myosin light chain kinase activity in an assay in which cal-

[2] A. M. Edelman, K. Takio, D. K. Blumenthal, R. S. Hansen, K. A. Walsh, K. Titani, and E. G. Krebs, unpublished observations.

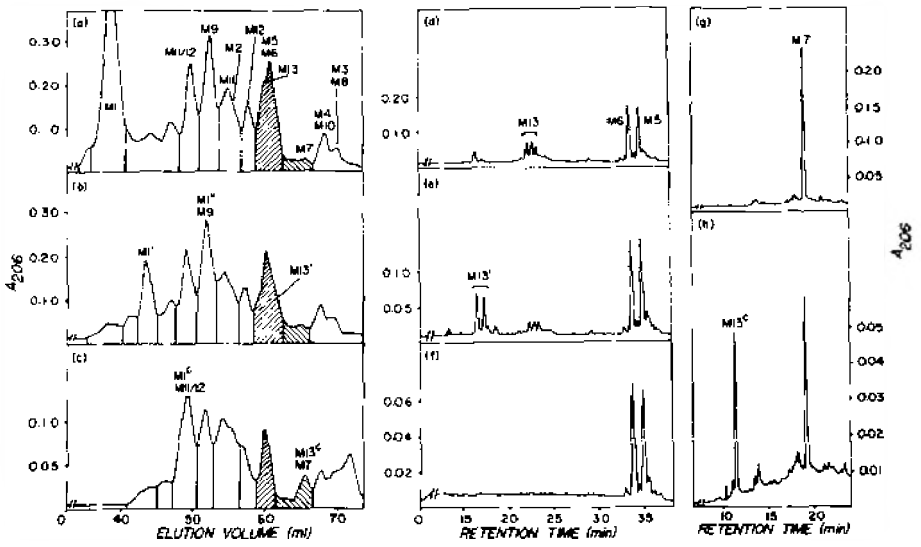

FIG. 11. **Separation of cyanogen bromide fragments of intact myosin light chain kinase, T_α/T_β, and $C_\beta35$.** CNBr cleavage was performed as described under "Experimental Procedures." Peptides M1 through M13 in *panel a* are numbered in order from the amino terminus of intact myosin light chain kinase (16). *Panels a–c:* primary separation in 6 M guanidine HCl, 10 mM phosphate, pH 6, on three TSK gel filtration columns (one G3000SW and two G2000SW, each 7.5 × 600 mm) connected in series at a flow rate of 0.5 ml/min. *Vertical lines* indicate pooled fractions. *Panels d–h:* further fractionation of selected pools from TSK gel filtration on a SynChropak RP-P (4.1 × 250 mm) column at a flow rate of 2 ml/min with gradients formed using solvents A (0.1% trifluoroacetic acid in H_2O) and B (0.08% trifluoroacetic acid in acetonitrile). *Panels d–f:* fractionation of pools containing M5, M6, and M13 or M13′ (▨) from *a–c*, respectively. The column was equilibrated at 10% B and eluted with a linear gradient to 40% B in 40 min. *Panel g*, a mixture of pools containing M7 (▩) from *a* and *b*. *Panel h:* the corresponding fraction containing M7 and $M13^c$ (▩) from *panel c*. For *panels g* and *h* the column was equilibrated at 100% A and eluted with a linear gradient to 60% B in 30 min. *a* and *d*, intact enzyme (0.95 mg); *b* and *e*, a 1.8/1.0 mixture of T_α and T_β (0.45 mg); *c*, *f*, and *h*, $C_\beta35$ (0.2 mg); *g*, intact enzyme and T_α/T_β combined (M7) pools.

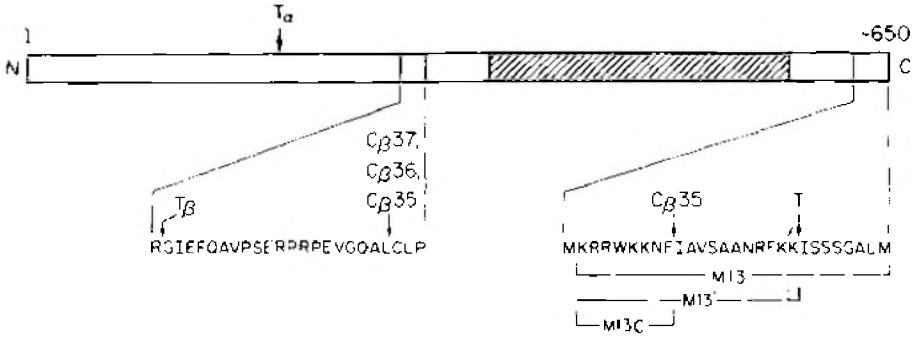

FIG. 12. **Schematic model of myosin light chain kinase showing amino- and carboxyl-terminal sequences of products of limited tryptic and chymotryptic cleavage.** *Bar* represents the entire molecule (~650 residues). *Arrows* indicate points of cleavage that yield the fragment indicated above the arrow. Carboxyl-terminal tryptic cleavage for T_α/T_β occurs at either the *solid* or the *dashed arrow*. The *hatched section* indicates the approximate location of the catalytic domain (see "Results"). M13, M13′, and $M13^c$ are the carboxyl-terminal cyanogen bromide peptides derived from intact enzyme, T_α/T_β, and $C_\beta35$, respectively. Sequences are in the one-letter code for amino acids.

modulin is subsaturating (15). 2) Proteolyzed forms of the enzyme ($C_\beta37$, $C_\beta36$, $C_\beta35$) differing greatly in their abilities to be activated by calmodulin (Table II) had identical amino termini. 3) The calmodulin-independent form ($C_\beta35$) was found to be missing 19 of its carboxyl-terminal 27 residues while in calmodulin-dependent forms (T_α/T_β) this region was largely intact (Figs. 11, 12). 4) CNBr digests of the calmodulin-dependent enzyme forms (native and T_α/T_β) inhibited calmodulin activation with K_i values of 4.5 and 1.3 nM, respectively, indicating that peptides M13 and M13′ were able to bind calmodulin with high affinity in an unfractionated digest (15). However, when the calmodulin-independent enzyme described here ($C_\beta35$) was subjected to CNBr cleavage and tested, there was little inhibition of calmodulin activation (K_i = 170 nM) suggesting that the octapeptide $M13^c$ does not retain the principal determinants required for calmodulin binding. The small degree of inhibition that was observed could be accounted for by a slight contamination of $C_\beta35$ with a calmodulin-dependent form.

To date, there have been two other reports describing calmodulin-independent forms of skeletal muscle myosin light chain kinase. Tanaka *et al.* (11) used high concentrations of trypsin (trypsin/kinase: >1.0, w/w) to create a calmodulin-insensitive enzyme. In contrast, at low trypsin/kinase ratios

(0.001, Fig. 2; 0.03, Mayr and Heilmeyer (10)) the calmodulin-dependent forms T_α and T_β were observed. It is therefore likely that at high trypsin concentrations further degradation at multiple sites occurs. At the carboxyl terminus, this cleavage might occur at ... KRRWKKN ... which would be expected to lead to calmodulin-independent activity (Fig. 12). We have also observed that digestion at higher trypsin concentrations will eventually lead to the development of calmodulin-independent activity (data not shown). Srivastava and Hartshorne (13) described a 65,000-dalton chymotryptic fragment whose activity was not regulated by calmodulin and which could be phosphorylated (0.8 mol of ^{32}P/mol of fragment) by cAMP-dependent protein kinase. We have found that phosphorylation by cAMP-dependent protein kinase (1.2 mol of ^{32}P/mol of myosin light chain kinase, Ref. 9) quite probably occurs between the amino terminus of T_α and that of T_β (data not shown). Therefore, the fragment of Srivastava and Hartshorne could be similar in structure to T_α, but with a slightly longer amino terminus and shorter carboxyl terminus. Since their digestion was performed in the presence of calmodulin, whereas ours lacked it, different digestion products might be expected. Nevertheless, we have been unable to observe this 65,000-dalton chymotryptic fragment even when digestion was performed in the presence of Ca^{2+}-calmodulin (data not shown). Although differences in other reaction conditions such as salt concentration, presence of sulfhydryl reagents or chelators, and type of buffer employed could conceivably account for these differences, this appears not to be the case as we have observed no significant differences in fragmentation pattern with changes in these variables. We therefore, at present, have no explanation for these discrepant results.

All calmodulin-requiring enzymes discovered to date have been reported to be rendered calmodulin-insensitive by limited proteolytic digestion (reviewed in Ref. 45). The fact that degradation induces an activity seen in the absence of calmodulin, implies that a calmodulin-dependent enzyme in its resting state is "internally inhibited" and that either proteolysis or calmodulin binding can relieve the enzyme of this inhibition. Once proteolyzed in this fashion (*e.g.* $C_\beta 35$), the enzyme would therefore be incapable of any further responses to calmodulin, that is, it would be calmodulin-independent. The mechanism of activation of the cyclic nucleotide-dependent protein kinases by cAMP or cGMP could be thought of in an analogous manner. That is, the binding of the cyclic nucleotide either causes the dissociation of the inhibitory (R) subunits in the case of cAMP-dependent protein kinase, or in the case of cGMP-dependent protein kinase, causes a conformational change in the enzyme relieving it of a resting inhibition.

To explain the different modes of interaction with calmodulin of the chymotryptic fragments ($C_\beta 37$, $C_\beta 36$, $C_\beta 35$) we propose that between the catalytic and calmodulin-binding domains there is an "inhibitory" domain. Thus it would appear that $C_\beta 36$, a form with a poor apparent affinity for, but complete dependence on, calmodulin has lost some of its calmodulin-binding domain but still retains its inhibitory domain. Proteolysis of $C_\beta 36$ to yield calmodulin-independent $C_\beta 35$ may involve either the removal of part of the inhibitory domain or a loosening of the tertiary structure thereby removing the inhibitory domain from a proximity to the active center. Several aspects of the purification of $C_\beta 35$ are consistent with the latter possibility. It was observed that during purification, the relative proportion of activity found in the absence of calmodulin rose (from about 50% to 100%) even though proteolysis had been terminated after only 20 min (Table III, Figs. 8 and 9). This can be explained if following proteolysis the enzyme underwent a slow transition (during the purification) from a constrained "inhibited" conformation to a less constrained "non-inhibited" conformation. Furthermore, if this were the case, then the apparent calmodulin-dependent activity would not be recovered in any fractions from the affinity column. Fig. 9 shows this to be the case. Also a transition to a less constrained form would be expected to result in an increased asymmetry to the fragment, an idea supported by the observation that the calmodulin-independent activity eluted from the HPLC gel filtration column slightly in advance of the calmodulin-dependent activity. Also, only a single peak of A_{280} absorbing material eluted from the HPLC column and throughout this peak, $C_\beta 35$ was the only protein species apparent by SDS-PAGE (data not shown). In a replicate digestion and purification of $C_\beta 35$, the same overall pattern was repeated (data not shown).

The calmodulin-dependent tryptic-fragments T_β or T_α/T_β showed modest decreases in the ability to be activated by calmodulin (Table II) and also were found to be missing the carboxyl-terminal 8 or 9 residues (Fig. 12). It is therefore conceivable that these residues participate in calmodulin binding. Nevertheless, M13 minus these carboxyl-terminal residues and M13 in its complete sequence (lacking only carboxyl-terminal homoserine) have been chemically synthesized and, if anything, the former shows a slight increase in affinity for calmodulin.[3] This apparent inconsistency may be due to differences in the precise way the peptide and intact enzyme interact with calmodulin (suggested also by the studies of Blumenthal *et al.* (15)). Alternatively the tryptic fragments may be slightly defective in the ability to convert binding into activation.

Finally, since the calmodulin-dependent tryptic fragments have the carboxyl-terminal sequence IAVSAANRFKk whereas these residues are missing from the calmodulin-independent form ($C_\beta 35$), this sequence would appear to contain critical determinants for calmodulin binding. The possible importance of these residues for this interaction is currently under active investigation.

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